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Mammalian Cell Culture Assay to Quantitate Chemically Induced Aneuploidy Use of a Monochromosomal Human/Mouse Cell Hybrid

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A MAMMALIAN CELL CULTURE ASSAY TO QUANTITATE CHEMICALLY INDUCED ANEUPLOIDY: Use of a Monochromosomal Human/Mouse Cell Hybrid

by

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16. ABSTRACT

A short-term assay utilizing a human/mouse monochromosomal hybrid cell line R3-5, to detect chemically induced aneuploidy in mammalian cells is described. A single human chromosome transferred into mouse cells was used as a cytogenetic marker to quantitate abnormal chromosome segregation following chemical treatment. The human chromosome present in the mouse cells can be readily identified by differential staining procedures. The frequency of cells containing 0 or 2 human chromosomes in the progeny of chemically-treated monochromosomal hybrid cells provided a direct measure of aneuploidy. We tested the sensitivity of the proposed system with three model chemicals (Colcemid, cyclophosphamide and benomyl) known to induce numerical or structural changes in chromosomes. The frequency of an abnormal segregation of the human chromosome was found to be dose dependent and consistently higher than controls. This system has the capability to detect gain as well as loss of a chromosome resulting from nondisjunction or other mechansisms leading to aneuploidy.

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INTRODUCTION

There is an increasing body of evidence to show that a significant proportion of human disease burden may be caused by exposure to environmental chemicals (Review in: Hiatt et al. 1977). Among the adverse health effects produced by these chemicals, genotoxic insult is perhaps the most serious. An exposure to toxic chemicals may produce genetic alterations in somatic and/or in germ cells. The genetic damage in somatic cells may lead to cancer or myriad of other diseases, but the effects are limited only to the individuals exposed to the harmful chemicals. The genomic changes in the germ cells, on the other hand, may be transmitted to the successive generations and become a legacy of human heritage. Cytologically observable chromosomal changes represent the most important category of geneticaly-defined human diseases. A variety of chromosomal aberrations such as deletions, duplications, inversions, translocations and numerical alterations in chromosomes (aneuploidy) have been implicated in human diseases (Hook 1983). Aneuploidy arising through non-disjunction during gametogenesis represents a single largest category of chromosomal aberrations leading to human birth defects (Hook, 1983).

Heteroploidy in cancerous cells has also been documented for sometime and non random chromosomal changes in association with cancer has been reported (Levan and Mitelman 1977). Although the role of aneuploidy in initiating carcinogenesis has not been established, it has been suggested that genomic alterations may predispose individuals to a greater risk of developing cancer (Arlett and Lehman 1978, Levan and Mitebman 1977, Bloom, 1972 and Tsutsui et al. 1983).

Aneuploidy in chemical mutagenesis has been emphasized relatively recently and only a limited amount of work has been reported in this direction (Zimmermann et al. 1979). Most commonly used biological systems to detect chemically induced aneuploidy involve fungi (de Bertoldi et al. 1980), yeast (Parry et al. 1981), and insects (Liang et al. 1983 and Zimmering 1982). However, mitotic poisons are known to have different

effects in lower and higher eukaryotes (Haber et al. 1972) and this difference has been demonstrated to be due to different target sites on microtubule (Heath 1975; Review in Bond and Chandley 1983). In the mammalian cells, total chromosome count following the exposure of cells to the test chemicals, has been used to assess the abnormal chromosome segregation at mitosis (Hsu et al. 1983; Danford, 1983 and Tsutsui et al. 1983). Such assays are hampered by the fact that cultured cell lines inherently contain a variable number of chromsomes and it is difficult to assess the loss or gain of individual chromosomes.

In this report, we have described a test system in which aneuploidy is assessed by the abnormal segregation of a single human chromosome present in a human/mouse somatic cell hybrid. A human chromosome present in mouse cells can be easily distinguished by differential staining procedures and serves as a cytogenetic marker. Progeny of a monochromosomal hybrid, in general, should contain a single human chromosome. The cells containing two or no human chromosome would result from non-disjunction. Thus, abnormal segregation resulting from the effect of chemicals can be detected from the frequency of cells containing two or 0 human chromosomes. In the present studies, we have used three chemicals, Colcemid, Benomyl and Cyclophosphamide previously known to induce aneuploidy to evaluate the proposed system. The system can be extended in the future, for the genotoxic evaluation of any chemical.

MATERIALS AND METHODS

Cell Line and Growth Conditions.

A microcell hybrid R3-5 between human and mouse cells containing a single human chromosome (monochromosomal) was employed to assess chemically induced aneuploidy. The R3-5 cells containing human chromosome 2 were produced by a two-step process involving gene transfer and microcell fusion (Dhar, et al. 1984). Details of the experiments on the production of R3-5 cells will be published elsewhere (R.S. Athwal, manuscript in preparation), while the properties pertinent to the present studies will be given here.

The human chromosome 2, present in R3-5 cells carries a selectable marker Ecogpt (Mulligan and Berg, 1981). Ecogpt is a cloned bacterial gene that codes for an enzyme xanthine guanine phosphorihosyl transferase (XGPRT), analogous to mammalian enzyme hypoxanthine guanine phosphorihosyl transferase (HPRT). XGPRT catalyses the formation of XMP from xanthine in the salvage pathway of purine biosynthesis, an activity normally not present in the mammalian cells. The cells carrying Ecogpt are capable of growth in the medium containing mycophenolic acid and xanthine, while the cells without Ecogpt cannot grow in this medium. Using this selection procedure (Mulligan and Berg, 1981), Ecogpt was first transferred to human cells (MGH-1) to produce a series of cell lines each carrying Ecogpt integrated into a different chromosome. Cells with Ecogpt integrated into chromosome 2 were then used as microcell donor to transfer this chromosome to mouse cells (R.S. Athwal, unpublished results).

Cultivation R3-5 cells in the medium containing mycophenolic acid and xanthine assures the presence of chromosome 2 in every cell at the time of treatment with chemical. The cells which may have lost the human chromosome would not survive in the selective medium. Human chromosome 2 being submetacentric, is morphological distinct from the mouse chromosomes and can be readily identified.

Figure 1. An outline of the experimental protocol to study chemically induced aneuploidy

Monochromosomal Hybrid Cells R3-5
Cultured in the selective medium (MX)

Plate 0.5×10^6 cells per T25 Flask
in non-selective medium (DME)

24 hours

Aspirate medium, Rinse cells with PBS
Add fresh medium (DME) and test chemical

24 hours

Remove chemical, Rinse cells with PBS
Add fresh medium

24 hours

Colcemid (.2 μg per ml)

2 hours

Cytogenetic Analysis

The R3-5 cells were routinely cultured at 37°C, in 10% CO_2, in Dulbeco's modified Eagle's medium supplemented with 10% fetal calf serum (DME), containing mycophenolic acid and xanthine (MX). Although, growth in the selective medium assures that all the cells contain the transferred human chromosome, the cell line was periodically analyzed cytologically for confirmation.

Experimental Protocol.

A schematic outline of the experimental protocol is given in Figure 1. For each experiment 0.5 x 10^6 cells were plated in T25 flasks in non-selective medium (DME), 24 hours prior to the chemical treatment. Cultures were rinsed twice with PBS and fed with DME containing the test chemical. After exposure to a chemical for 24 hours, cultures were rinsed twice with PBS, fed with fresh DME and allowed to grow for another 24 hours and then harvested for cytogenetic analysis.

Cytogenetic Analysis.

Following the exposure to the test chemical, cells were arrested in mitosis with colcemid (0.2 μg/ml) for 2 hours. Mitotic cells harvested by shaking the flasks were swollen in hypotonic solution (0.075M KCl) for 15 minutes and then fixed in three changes of fixative (3 methanol:1 acetic acid). Metaphase spreads prepared by air drying were stained either by Giemsa-11 (G-11) method (Bobrow and Gross, 1974) or with Hoechst 33258 (Hilwig and Gropp, 1972) to distinguish between human and mouse chromosomes.

For staining with G-11, one day old slides were soaked in water at 37°C for 30 minutes and then rinsed in sodium phophate buffer (pH 11.3) for 1 minute. Following the pre-treatment, slides were stained in the staining solution prepared in Na_2HPO_4 (0.05M) buffer (pH 11.3) for 3-4 minutes at 37°C. The staining solution was prepared fresh by mixing 240 μl of Azur B (1% in H_20) and 200 μl of Eosin (0.25% in methanol) into 20 ml of buffer.

Alternatively, in some experiments the metaphase spreads were also stained with fluorescent dye Hoechst 33258. The slides were pretreated with NaOH (0.07N) dissolved

in 30% ethanol for 1-2 minutes and rinsed three times in 70% ethanol to stop denaturation. Following the ethanol treatment, the slides were rinsed twice in PBS and stained for 15 minutes in Hoechst 33258 (0.05 µg/ml) dissolved in PBS. The stained slides were rinsed with distilled water, air dried, and wet mounted in 0.08M sodium phosphate (0.12M), sodium citrate buffer (pH4.1). The metaphase spreads were examined under fluorescence microscope (Zeiss) equipped with epi-illuminator to give excitation and reflector combination of 365nm and 420nm, respectively. At least 200 metaphase were scored for each treatment of the chemical.

Chemicals and Concentration.

The concentrations tested for each chemical are given in Table 1. Three chemicals, i.e., Cyclophosphamide (Cy), Colcemid, and benomyl, known to induce chromosome breaks or interfere with cell division were used as model agents to test the proposed system. Concentrations for each chemical selected on the basis of cytotoxicity included at least one dose above and 1-3 doses below the toxicity level. The toxicity was determined by exposing cells (10^6), seeded in T 25 flasks, to different concentrations of a chemical for 24 hours. The inhibition of growth, determined by counting viable cells following chemical treatment for 24 hours, was used as a measure of toxicity. Since the toxicity of a chemical is influenced by cell density at the time of treatment, similar experimental conditions were used for toxicity analysis as for the induction of aneuploidy.

Colcemid was purchased as 10 µg/ml solution in Hank's BSS (Gribco) and diluted further in the medium to achieve the appropriate concentration. Cyclophosphamide (Sigma) was disolved in water, while benomyl (Du Pont de Nemours, Wilmington, DL., USA) was dissolved in dimethyl sulfoxide (DMSO).

Colcemid and benomyl do not require metabolic activation to manifest their genotoxic activity. However, both are active even in the presence of S-9 fraction of rat liver microsomes. Cy, on the other hand, produces genotoxic effects only when

metabiolically activated. Therefore, in the studies reported here, Cy was tested with and without metabolic activation using S-9 fraction of rat liver microsomes (McCann and Ames 1977). The data on Cy without metabolic activation served as a negative chemical control.

RESULTS

After chemical treatment, individual metaphases of R3-5 cells were analyzed to estimate the total number of chromosomes and to determine the number of human chromosomes present in each cell. Normally, the number of mouse chromosomes present in R3-5 cells varies from 46-56 (Fig. 2). Following the chemical treatment, the R3-5 cells containing the chromosome number within the range of parental genome were classified as diploids; while the cells with twice or more the number of R3-5 chromosomes were classified as polyploids. Only the cells with diploid number of chromosomes were scored for the segregation of human chromosomes. Human chromosome present in mouse cells was identified by G-11 or Hoechst staining methods (Fig. 2). With G-11, human chromosomes stained blue, whereas mouse chromosomes stained magenta (Fig. 2). In the case of Hoechst 33258, the human chromosome present among mouse chromosomes was identified by the lack of characteristic centromeric fluorescence of the mouse chromosomes (Fig. 2). The data on the induction of genomic alterations are listed in Table 1. Since the cells were analyzed for aneuploidy and polyploidy, the results for these end points are presented separately.

Aneuploidy.

Normally, the progeny of a monochromosomal hybrid should contain only a single human chromosome. The cells containing 0 or 2 human chromosomes were considered to result from aberrant segregation of the human chromosome and were pooled to calculate aneuploidy. In comparison to the control, the frequency of aneuploid cells increased in a dose dependent fashion after the treatment with Colcemid and benomyl (Table 1).

In case of cyclophosphamide, increase in the number of aneuploid cells occurred only when used with metabolic activation. Without metabolic activation, there was no difference in the treated or untreated cultures. Even with metabolic activation, Cy at 10 µg/ml had no effect on the frequency of aneuploid cells. The number of aneuploid

cells increased from 4 percent in control to 8 percent at 15 μg/ml of Cy and remained the same at 20 μg/ml. It was interesting to note that the increase in the number of aneuploid cells, in the case of Cy, was observed only in the class of cells that had lost the human chromosome (0-chromosome class), rather than the one with an extra chromosome (2 chromosome class). On the other hand, in case of Colcemid and benomyl, the number of cells that gained or lost the human chromosome increased simultaneously with an increase in the concentration (Table 1). These results indicate that Cy may have induced aneuploidy by a mechanism different than that for colcemid and benomyl.

Induction of Polyploidy.

The data given in Table 1 show a dose dependent increase in the frequency of polyploid cells after treatment of the hybrid cells with Colcemid and benomyl. At concentration levels up to 0.01 μg/ml of Colcemid, the number of polyploid cells did not change, while the frequency of aneuploid cells increased (Table 1). Similarily, at the lowest dose of benomyl (1.5 μg/ml), although the frequency of aneuploid cells increased from 2.04% in control to 16.75% in the treated cells, no change in the number of polyploid cells was observed. However, at higher doses of both Colcemid and benomyl, the frequency of polyploid cells increased at a much faster rate than the aneuploid cells. This dose dependent difference in the aneuploidy and polyploidy perhaps may be due to the relative increase in spindle disruption resulting from the saturation of the target molecules at higher concentrations of Colcemid and benomyl (Table 1). The differential increase in the number of polyploid and aneuploid cells at different doses was reflected in the ratio of polyploid/aneuploid (P/A) cells. At low doses (0.01 μg/ml of colcemid and 1.5 μg/ml of benomyl), P/A ratio was lower than that in the control, whereas at high doses, as the number of polyploid cells increased, a corresponding shift in P/A ratio (Fig. 3) occurred. A high P/A ratio of 0.98, observed in the control cultures for benomyl was due to the presence of equal number of polyploid and aneuploid cells (Fig. 3).

Cy had no effect on the frequency of polyploid cells either with or without metabolic activation (Table 1). In comparison with the controls, no change in P/A ratio was observed for Cy without metabolic activation, while a decrease in the ratio occurred when the chemical was used with metabolic activation (Fig. 3). We interprete these results to show that Cy does not interfere with the cell division. Cy is a known clastogenic agent (Goetz et al, 1980) and data given in Table 1 show that frequency of cells with chromosome aberrations involving the human chromosome increase with the dose. The increase in the frequency of cells with chromosome aberrations correlated with the chromosome loss in case of metabolically activated Cy. These results show that Cy, perhaps induced aneuploidy by direct effect on the chromosome rather than interfering with cell division.

DISCUSSION

Data presented in this paper show that a human/mouse hybrid cell line may provide a useful tool for evaluating the potential of environmental chemicals to induce aneuploidy. The human chromosome 2 present in R3-5 cells is easily distinguishable from the mouse chromosomes, thus provides an excellent cytogenetic marker (Fig. 2). We would like to emphasize that, this test system measures the abnormal segregation of a single chromosome rather than aneuploidy in general. Chemically induced abnormal distribution of a single chromosome to daughter cells at mitosis is used as an index for aneuploidy.

Aneuploid condition is characterized by the gain (2n+1) or loss (2n-1) of a chromosome of the complement. The gain or loss of a chromosome at mitosis usually is a complementary consequence of nondisjunction. Loss, however, could also occur due to the failure of a chromosome to incorporate into the daughter nucleus by other mechanisms such as anaphase lagging, lack of centromere separation or chromosome breaks (Bond and Chandley, 1983; Vig, 1984). The proposed system has the capability to detect gain as well as loss of a chromosome induced by the test chemical.

Colcemid and benomyl bind to the same target molecule (Quinlan et al, 1981), thus producing similar effects. Although, the number of cells for the loss and gain of the human chromosome increased simultaneously with the dose of Colcemid and benomyl, the frequency of cells with chromosome loss was consistently high at all doses. These result show that more than one mechanism may account for chromosome loss, while the gain may always result from non-disjunction. In contrast, cyclophosphamide induced only the loss of the chromosome which indicates a different mechanism of action for this compound. Cy is a known clastogenic agent (Goetz et al., 1980) and chromosome loss observed in this case may be due to chromosomal breaks. In fact, in our analysis, an increase in the frequency of cells with complex rearrangements involving human

chromosome corresponded with frequency of chromosome loss with CY (Table 1). Therefore, the aneuploid condition observed in this case is attributed to the clastogenic effect of Cy rather than the interference with the cell division. This notion is further supported by the fact that increase in the frequency of polyploid cells did not occur with Cy (Table 1).

The P/A ratio as discussed in results may provide an index to detect the potential of a chemical to induce aneuploidy and determine its mode of action. As apparent from our results, the P/A ratio remains same, both in the treated and control cultures, when a chemical does not have any effect on the cell division or chromosomes (Fig. 3). An increase in the P/A ratio with the concentration would indicate interference with the cell division, while a decrease indicates the effect of the test chemical on the chromosome rather than cell division (Fig. 3).

Other mammalian cell culture systems to assay for chemically induced aneuploidy include the use of continuous or early passage diploid Chinese hamster (Hsu, et al. 1983; Onfelt and Klasterka, 1983; and Danford, 1984; Cox, 1973) or Syrian hamster cells (Tsutsui et al, 1983). In these systems, complete mitotic arrest (Onfelt and Klasterka, 1983), total chromosome count (Danford, 1984; Tsutsui, et al. 1983), or anaphase abnormalities such as multipolar spindle and lagging chromosomes (Hsu, et al. 1983) have been used as criteria to assess the ability of chemicals to interfere with cell division. Total chromosome count, in addition to being tedious and time-consuming may not provide complete information. A cell may simultaneously lose and gain a chromosome in two different homologous pairs and still retain the parental chromosome number. In this case, although aneuploidy has occurred, the chromosome number remains unchanged.

Another system similar to ours in principal is the use of human male lymphocytes (Tenchini, et al. 1983). In this system, human Y chromosome was used as a marker to assess aberrant segregation following chemical treatment. The occurrence of YY mitosis was considered to result from a non-disjunctional event. However, the frequency of YY

cells was rather low. In addition, this system detected only chromosome gain and not the loss.

In the hybrid cell system, the ease to identify human chromosome greatly reduces error in scoring. In addition, it eliminates the need for laborious chromosome counting or karyotypic analysis. One may contend that a human chromosome present in mouse cells may not obey the same rules at cell division as the parental mouse chromosomes, and therefore may be relatively unstable in the foreign environment. The absence of aneuploid cells in cultures treated with Cy without metabolic activation, clearly shows that abnormal segregation of the human chromosome is not a random non-specific phenomenon, but a function of chemicals which interfere with cell division or cause chromosome breaks. The relative unstability of the human chromosome may rather be useful in making the assay more sensitive.

The future direction of this investigation is to develop a system to detect aneuploidy by selection and colony formation. Like mammalian HPRT, E. coli XGPRT can also transfer purine analogue 6-thioguanine (G-TG) to form corresponding monophosphate which is toxic to the cells. Therefore, if R3-5 cells are cultured in the medium containing 6-TG, only the cells which may have lost the human chromosome would survive. This back selection procedure thus provides an assay system to determine the frequency of cells with chromosome loss following treatment with a test chemical.

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FIGURE LEGENDS

Figure 2. **Metaphase spreads of monochromosomal hybrid R3-5**

(A) Stained with G-11, single human chromosome can be identified by difference in color and chromosome morphology.

(B) Stained with Hoechst 33528. Human chromosome is distinguishable by the lack of intense fluorescence of centromeric heterochromatin, characteristic of mouse chromosomes.

(C) Metaphase of R3-5 cells containing two human chromosomes following treatment with colcemid.

(D) A polyploid cell containing two human chromosomes.

Figure 3. **Ratio of polyploid/aneuploid (P/A) cells plotted against the concentration of each chemical.**

(A) Colcemid, (B) Benomyl, (C) Cyclophosphamide with metabolic activation and (D) Cyclophosphamide without metabolic activation.

Figure shows a dose-related increase in P/A ratio for Colcemid and benomyl, a decrease with metabolically activated cyclophosphamide and no change with cyclophosphamide without metabolic activation.

TABLE 1

Number of cells containing 1, 2 or 0 human chromosomes and polyploid cells following treatment with test chemicals.

Chemicals & Conc. (μg/ml)	No. of Metaphases Examined	Cells with 1, 2, and 0 Human Chromosomes 1	2	0	Percent Aneuploid Cells[a] (A)	Percent Polyploid Cell (P)	Ratio P/A[b]	Percent Cells[c] with Chromosome Aberrations
Cyclophosphamide (+S9)								
0	200	192	4	3	3.51	0.5	0.14	0
10	200	191	5	3	4.02	0.5	0.12	0.5
15	200	192	3	4	3.51	0.5	0.14	0
20	200	193	3	3	3.03	0.5	0.16	0.5
Cyclophosphamide (-S9)								
0	200	191	3	5	4.02	0.5	0.12	0
10	200	190	3	6	4.52	0.5	0.11	3
15	200	183	5	11	8.04	0.5	0.06	4
20	200	182	5	12	8.54	0.5	0.06	6
Colcemid (-S9)								
0	200	191	4	4	4.02	0.5	0.12	0.5
0.005	200	185	7	7	7.04	0.5	0.07	0.5
0.01	200	180	9	10	9.55	0.5	0.05	0
0.02	200	139	12	14	15.75	17.5	1.11	0.5
0.05	200	96	16	20	27.27	34.0	1.25	0
0.1	200	86	11	14	22.52	44.5	1.98	0.5
Benomyl (-S9)								
0	200	192	1	3	2.04	2.0	0.98	0.5
1.5	200	164	13	20	16.75	2.0	0.12	0.5
3.0	400	289	35	39	20.39	9.2	0.45	1
7.5	300	161	26	30	25.81	27.7	1.07	1
15	300	142	24	27	26.42	35.7	1.35	4

[a]The percent of Aneuploid cells was calculated by adding cells with 0 and 2 human chromosomes and taking diploid and aneuploid cells for total; (i.e., excluding polyploid cells).

[b]Ratio of polyploid/aneuploid cells was calculated from the percent of polyploid and aneuploid cells.

[c]Cells with chromosomal aberrations involving human chromosome were counted in the scored metaphases.

Note: Aneuploidy in this system refers to the gain or loss of the human chromosome in R3-5 cells, while polyploidy refers to the increase in the total number of chromosome by a factor of 2 or more.